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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Nancy T. Chang

Serial No.: 659,339

Filed: October 10, 1984

Title: CLONING AND EXPRESSION OF HTLV-III DNA

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Washington, D.C. 20231, on 5-14-86

Hamilton, Brook, Smith & Reynolds

Ellen Ritrovato
Signature

5-14-86
Date

DECLARATION OF NANCY T. CHANG

The Honorable Commissioner
of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Nancy T. Chang, of 7405 Brompton St., Houston, Texas 77025, declare:

1. I am an inventor of the subject matter described and claimed in the above-identified application. When the invention was made, I was

Affidavit Exhibit 2
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Interference No. 103,659

Associate Research Director in Molecular Biology at Centocor, Incorporated, Malvern, Pennsylvania (Centocor), assignee of the subject application. Currently, I am an Associate Professor of Medicine at Baylor College, Houston, Texas.

2. At the time the application was filed, Dr. Robert C. Gallo and Dr. Flossie Wong-Staal were not designated co-inventors when, in fact, they were co-inventors and should be so designated.

3. The above-identified application discloses and claims methods for cloning and expressing subgenomic fragments of HTLV-III cDNA; HTLV-III cDNA fragments and immunoreactive HTLV-III polypeptides encoded thereby; and methods of detecting antibody against HTLV-III employing the polypeptides.

4. The experimental work described in the application began at Centocor upon receipt of genomic HTLV-III DNA from the laboratories of Dr. Gallo and Dr. Wong-Staal. Dr. Gallo and Dr. Wong-Staal supplied a recombinant phage (designated λBH 10) consisting of the genomic HTLV-III cDNA recombined with a phage vector. The HTLV-III cDNA insert was excised from λBH 10 and fragmented and the subgenomic fragments were cloned and expressed in host cell systems as described in the application. All of the experimental work described in the application was done at Centocor, either by me or by laboratory assistants working under my direction and supervision. However, at various times before the experimental work and during its progress, Dr. Gallo, Dr. Wong-Staal and I discussed the strategy

for the cloning and expressing of the viral cDNA. The experimental work proceeded along the lines we discussed; thus Dr. Gallo and Dr. Wong-Staal contributed signficantly to the cloning and expression of the HTLV-III cDNA.

5. On August 22, I prepared a document which described the experimental work accomplished up to that time. The document was sent to Centocor's patent law firm, Hamilton, Brook, Smith & Reynolds (HBS&R), as an "invention disclosure" (Exhibit A). Because all of the work described in the "invention disclosure" document was done at Centocor and because of my incomplete understanding of the law of inventorship, I did not designate Dr. Gallo or Dr. Wong-Staal as "inventor" on this document.

6. Subsequently, Centocor decided to have a patent application prepared and filed by HBS&R. Because of the imminent publication of an article disclosing work relating to the invention, there was great urgency to file the application. On October 8, 1984, I met with Centocor's patent attorneys to supplement information contained in the "invention disclosure" document (Exhibit A) for completing of a patent application. At this meeting, all of my time was devoted to explanation and discussion of the highly technical and complex subject matter necessary to prepare the application. The subject Application, Ser. No. 659,339 was filed on October 10, 1984.

7. On January 23, 1985, a continuation-in-part application was filed to cover additional experi-

mental work which had been done since the earlier application was filed. The inventorship error was repeated.

8. The possibility of an error in inventorship was first raised by Dr. Gallo in a letter to me dated July 25, 1985 (Exhibit B). Shortly thereafter, Centocor management initiated an investigation into the facts surrounding the invention and authorized HBS&R to do the same (Exhibit C). After a preliminary investigation, Centocor management made a tentative response to Dr. Gallo on September 16, 1985 (Exhibit D). However, pursuant to Centocor's stated desire to have the patent legally drawn a thorough investigation was made. During this investigation, I informed HBS&R of the full extent of Dr. Gallo's and Dr. Wong-Staal's collaboration with me regarding conceptual aspects of the claimed subject matter. After consideration, HBS&R concluded that Dr. Gallo and Dr. Wong-Staal should be designated as co-inventors because of their conceptual contributions.

9. My earlier failure to indicate the contributions of Robert C. Gallo and of Flossie Wong-Staal was unintentional.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001

of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Nancy T. Chang

Nandy T. Chang

Feb. 23, 1986

Date

EXHIBIT A

THE CHARACTERIZATION AND PRODUCTION OF HTLV-III GENES AND PROTEINS BY GENETIC ENGINEERING METHODS

Nancy T. Chang

Centocor, Inc.

244 Great Valley Parkway

Malvern, PA 19355

August 22, 1984

Nancy T. Chang
Aug. 27, 1984

8/27/

Witnessed by

Aug 27, 1984

Documentary Exhibit 5
CHANG ET AL.
Interference No. 103,659

Diagnostic and Vaccine Developments for AIDS

Human T Cell Leukemia Virus type III (HTLV-III), also named Lymphoadenopathy Virus (LAV), was isolated from the peripheral blood or lymphoid tissues of patients with Acquired Immune Deficiency Syndrome (AIDS). Recent studies of R. Gallo's group and of L. Montagnier's group indicated that the sera from over 80% of AIDS and pre-AIDS patients contain antibodies specific for the viral envelope and core proteins of HTLV-III. This and other evidence strongly suggests that HTLV-III is the cause of infectious AIDS, giving hopes that the diagnosis, preventive vaccine, and even therapy for AIDS can soon be developed.

Because AIDS can be transmitted through blood transfusions, an assay that detects HTLV-III infection is important not only for diagnosing patients but also for screening blood that might be contaminated with the virus. NIH and several commercial firms, including Centocor, are now developing immunoassay kits employing inactivated, disrupted HTLV-III as the solid-phase immunoadsorbent for the detection of antibodies against HTLV-III antigens in serum or blood.

Genetic Engineering Approach

Another approach for the detection of and the vaccination against HTLV-III infection is the employment of genetic engineering methods. In this approach, the proviral genes integrated into host cell DNA are molecularly cloned. The nucleotide sequence of the molecular cloned provirus is determined. The viral nucleotide sequence information will be directed to design and engineer HTLV-III-specific peptides and DNA probes using recombinant DNA technology or synthetic peptide chemical synthesis methods. These products are then explored for use in the diagnosis of HTLV-III infections by measuring specific antibody to the viral peptides or HTLV-III-specific RNA or DNA. The peptides, especially the gag and env related peptides may also be used as vaccines for the prevention of AIDS.

More specifically, the env and gag genes, which encode the envelope and core proteins of HTLV-III, respectively, are subcloned into various bacterial or mammalian expression vectors. These expression vectors contain all the necessary controlling elements for the production of the fused HTLV-III env gene in recombinant plasmids bearing host cells. Expression of the HTLV-III related peptide in the foreign host cells can be detected by binding of HTLV-III specific antibody in the AIDS patient serum or hyperimmune serum raised against purified virus. Although the env and gag products are of primary interest for diagnostic and vaccine purposes, the other two genes encoded by HTLV-III, pol and Px are important for understanding the biology of this retrovirus. These genes will be studied as well.

The genetic engineering approach offers a few advantages over the conventional one, which involves growing HTLV-III in cell cultures. For example, in the manufacturing process, because viral antigens are not infectious, working personnel are not exposed to the hazardous virus and the facility requirement will be less stringent than that for virus production. Also, the envelope and core proteins are the dominant immunoreactive viral antigens, immunoadsorbents with the purified viral proteins may offer more antibody-adsorbing capacity and higher sensitivity than those with whole virus. Immunoassays employing envelope and core proteins separately can detect antibodies against envelope and against core proteins. The antibody profile (concentrations and proportions) may reveal certain natures of the disease yet to be discovered. Furthermore, a protein vaccine using purified viral proteins (env or core gene product) will not have the risk of viral infectivity.

Centocor's First Footstep in HTLV-III Molecular Biology Work

As soon as we obtained the information in early May, 1984, that HTLV-III was isolated from AIDS patients and shown convincingly to be the cause of AIDS and that antibodies against HTLV-III antigens were found in over 85% of AIDS and Pre-AIDS patients. I decided to use the genetic engineering approach to develop diagnostic assays for AIDS. On May 10, 1984, Tse Wen Chang, Michael Wall and myself went to Biotech Corporation, Rockville, Maryland, to meet Dr. Robert Ting (Chairman of Biotech) to discuss the collaboration between Centocor and Biotech about coating polystyrene beads with inactivated disrupted HTLV-III. In that meeting, I expressed my interest to clone and

express HTLV-III genes and to use the expressed proteins for diagnostic and vaccine products. Dr. Ting was impressed with our expertise in Molecular Biology and introduced me to Dr. Flossie Wong-Staal, a key associate of Dr. Robert Gallo, with whom he had been collaborating on certain aspects of HTLV-III work. Our collaboration with the NCI group started on that day. We returned to Centocor with E. coli clones encoding segments of HTLV-II DNA. At that time, HTLV-III DNA had not been cloned.

The collaboration between Centocor and the NCI group went on very nicely. On July 5, we visited Dr. Wong-Stahl reporting our progress on HTLV-II and proposing our strategy on HTLV-III. We obtained λ clones harboring a segment of HTLV-III DNA on July 20, 1984. Our work on HTLV-III started on that day.

Centocor's Progress Update

We now have E. coli plasmid clones containing various portions or entire genome of HTLV-III. We have sequenced a segment (about 3500 base pairs long) of HTLV-III genome encoding most of the env gene. We have also cloned HTLV-III DNA in several expression host-vector systems and obtained several clones that can be induced to synthesize polypeptides encoded by the inserted HTLV-III DNA. Efforts are being made to test the reactivity of these polypeptides with antibodies from AIDS patients. When we identify clones that produce polypeptides demonstrating good reactivity with the antibodies, we will produce the polypeptide in large quantities and use it in immunoassay development. We also plan to clone and express the gag gene in a few weeks.

Plans are also being made to transfect mammalian cells with the E. coli cloned env and gag DNA's.

The Application of HTLV-III Related Peptides or Proteins

The viral envelope and core related peptides produced by the env and gag clones, either separately or combined, can be coated or conjugated noncovalently or covalently onto solid phase, such as PVC plate or polystyrene beads to be used as immunoadsorbent for antibodies against them. These solid phase immunoadsorbents are the key components in the immunochemical assays for HTLV-III-specific antibodies, using tracers such as goat anti-human immunoglobulin or protein A that are conjugated with radioactive isotopes such as ^{125}I, or enzymes such as peroxidase or alkaline phosphatase.

The proteins can also be used to prepare vaccine against HTLV-III, which should be useful for high-risk populations, such as homosexual males and recipients of frequent blood transfusions. The genetic engineered envelope and core proteins can also be used as an immunogen to prepare monoclonal or polyclonal antibodies. These antibodies can be employed in immunochemical assays for the detection of viral antigens in serum, blood, lymphocytes, or other tissues of AIDS or pre-AIDS patients.

The nucleotide sequences of HTLV-III env and gag genes yield information about the amino acid residue sequences of the envelope and core proteins.

Artificially synthesized segments of polypeptides according to the sequences may offer potential in diagnostic assays and in vaccines.

The cloned HTLV-III and its sequence can also be used to prepare DNA probes for the detection of HTLV-III RNA, proviral DNA, or encoded mRNA in the lymphocytes, or other tissues of patients.

EXHIBIT B

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20205
Building : 37
Room : 6A09
(301) 496-6007

July 25, 1985

Dr. Nancy Chang
Assistant Research Director
Molecular Biology
CENTOCOR
244 Great Valley Parkway
Malvern, PA 19355

Dear Nancy:

We are pleased that our collaborative efforts are making progress. Your synthesis of HTLV-III env gene products using our HTLV-III DNA clone is encouraging. We are beginning to use these in our larger NCI vaccine research development effort.

However, it has come to our attention that some time ago your organization filed a patent on the synthesis and uses of the expressed products from our HTLV-III DNA clones which were designated for collaborative research. We found out that our names are not included on the patent, despite the fact that your use of the clone was indispensable to your part of the effort.

We assume that this was an oversight. We would like to ask that your patent be modified and that our names be added to your patent application. We feel that a formal recognition of our contribution is integral and that the inclusion of our names is only fair.

Sincerely yours,

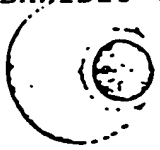
Robert C. Gallo, M.D.

RCG/PF/bj

cc Dr. Chabner
Dr. DeVita
Dr. Fischinger
→Dr. Harmison
Dr. Sliski
Dr. Wall ✓

Documentary Exhibit 6
CHANG ET AL.
Interference No. 103,659

EXHIBIT C



244 GREAT VALLEY PARKWAY. MALVERN. PA 19355. (215) 296-4488
TELEX: 834823 CENTOCORMARN
FAX: 215-644-7558

August 5, 1985

David Brook, Esquire
Hamilton, Brook, Smith & Reynolds
Two Militia Drive
Lexington, Massachusetts 02173

Dear David:

I will respond to Dr. Gallo at the National Institutes of Health upon my return, August 20, 1985. In the meantime please discuss this matter with Nancy Chang regarding the facts surrounding this invention.

I believe Dr. Gallo mixes up inventorship with contribution. This issue is politically sensitive and I may wish to compromise. I will also discuss this with Dr. Lawless at Du Pont. Du Pont is licensed by the government.

Sincerely,

Hubert J.P. Schoemaker for

Hubert J.P. Schoemaker, Ph.D.
President

HJPS:so'h
attachment
cc: Dr. Nancy Chang
Dr. Gregory Lawless

Documentary Exhibit 7
CHANG ET AL.
Interference No. 103,659

EXHIBIT D

CENTOCOR

244 GREAT VALLEY PARKWAY, MALVERN, PA 19355. (215) 296-4488
TELEX: 834823 CENTOCORMARN
FAX: 215-644-7558

September 16, 1985

Dr. Robert Gallo
National Institutes of Health
9000 Rockville Pike
Building 37
Room 6A09
Bethesda MD 20205

Dear Dr. Gallo:

I have in hand your letter of July 25, 1985 addressed to Dr. Nancy Chang regarding inventorship on the Centocor HTLV-III patents. There is no question that your collaboration was essential to the overall program and, as you know, we have on every occasion, made this fact clear.

In the case of the patent covering the assay development, our lawyers advised us that, under strict inventorship interpretation, your contribution should be referenced in the patent but that you should not appear as an inventor. These rules are quite contrary to the rules for authorship on scientific papers.

We wish to have the patent legally drawn. Anything to strengthen the patent is an advantage. If the lawyers feel your name should be added, we would be not only willing but anxious to have this accomplished.

I would be happy to discuss this matter with you or your representative or arrange to have our patent attorneys visit you in Washington. If you wish to talk to our attorney, please feel free to call David Brook of Hamilton, Brook, Smith & Reynolds directly at 617-861-6240. David does our patent work and his principal client is MIT. He is most qualified in the patent area.

Your work for the government and the community is outstanding. We have attempted to support you to our utmost in the past and will use our best efforts to support you in the future.

Yours very truly,

Hubert J.P. Schoemaker, Ph.D.
President

cc: D. Brook, Esq.
N. Chang, Ph.D.
H. Wall, Chairman

Documentary Exhibit 8
CHANG ET AL.
Interference No. 103,659

American Type Culture Collection
12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301)881-2600 Telex: 898-055 ATCCNORTH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

National Institutes of Health, National Cancer Institute
Building 37, Room 6A17
9000 Rockville Pike
Rockville, Maryland 20205
Attention: Dr. Flossie Wong-Staal

Deposited on Behalf of: National Institute of Health, National Cancer Institute

Identification Reference by Depositor:	ATCC Designation
λ EH-10 recombinant phage clone of HTLV-III in λg & Wes λB	40125
λ EH-5 recombinant phage clone of HTLV-III in λg & Wes λB	40126
λ EH-8 recombinant phage clone of HTLV-III in λg & Wes λB	40127

The deposits were accompanied by: ___ a scientific description ___ a proposed taxonomic description indicated above.

The deposits were received July 30, 1984 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

___ We will inform you of requests for the strains for 30 years.
X We will not inform you of requests for the strains.
___ The strains are available to the scientific public upon request as of

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same:

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above were tested March 4, 1987. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC: Bobbie A. Brandon
(Mrs.) Bobbie A. Brandon, Head, ATCC Patent Depository

Date: March 6, 1987
cc: James A. Oliff, Esq.

Form BP 4/9

P.3

NOV 22 '95 10:33AM ATCC

Documentary Exhibit 12
CHANG ET AL.
Interference No. 103,659

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail in an envelope addressed to: the Assistant Commissioner for Patents, Washington, D.C., 20231, on February 20, 1996.

Dated: May ___, 1996 By: _____
Eugene Moroz

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of : Nancy Chang et al.

Serial No. : 06/659,339

Filed : October 10,1984

For : CLONING AND EXPRESSION OF HTLV-III DNA

Assistant Commissioner for Patents
Washington, D.C. 20231

RENEWED PETITION UNDER 37 C.F.R. §1.182

Sir:

Attached is a REQUEST FOR RECONSIDERATION OF THE MARCH 29, 1996 DECISION DISMISSING APPLICANTS' PETITION PURSUANT TO 37 C.F.R. §1.182 TO ADD A REFERENCE TO A PRE-FILING DATE DEPOSIT.

Respectfully submitted,

MORGAN & FINNEGAN, L.L.P.

By: _____
Eugene Moroz
Reg. No. 25,237

Of Counsel:

William S. Feiler
M. Caragh Noone
MORGAN & FINNEGAN, L.L.P.
345 Park Avenue
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(212)758-4800
(212)751-6849 (FAX)

James C. Haight
NATIONAL INSTITUTES OF HEALTH
Office of Technology Transfer
Suite 325
6011 Executive Blvd.
Rockville, MD 20852
(301) 496-7056

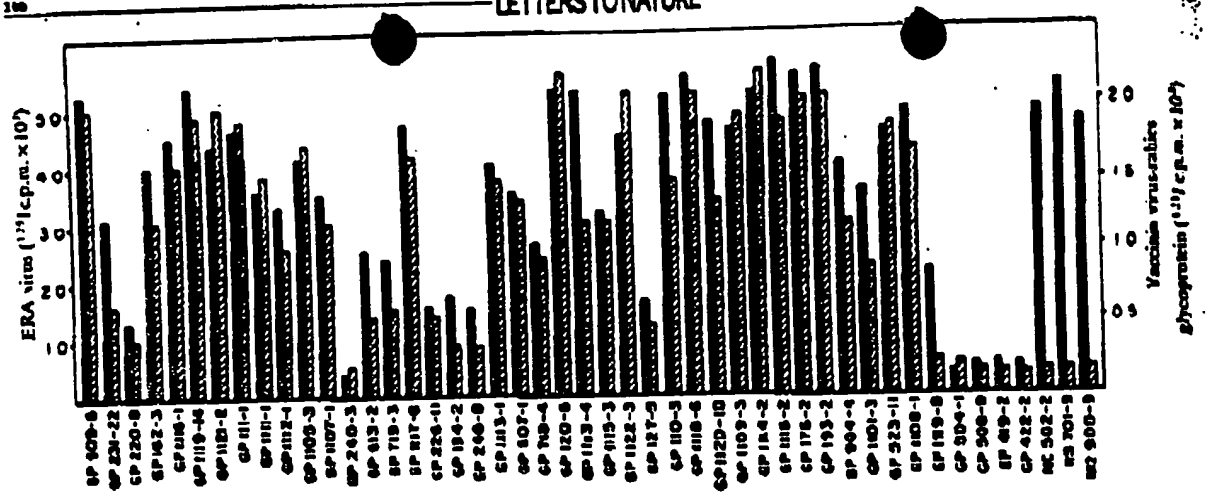

Fig. 3 Comparative binding of VVTGgRAB and ERA virus antigens with a panel of monoclonal antibodies. Solid bars, ERA virus; cross-hatched bars, VVTGgRAB virus.
Methods: Antigens (100 ng) were dried on microtitre plates and treated for 30 min with phosphate-buffered saline (PBS) containing 10% γ-globulin-free horse serum (Gibco). After draining, monoclonal antibody (1 : 1,000 dilution of ascites fluid; 25 μl) was added, incubated for 1 h at 37 °C and washed with PBS. Each well then received 25 μl of ^{125}I-labelled goat anti-mouse antibodies (30,000 c.p.m., specific activity, 0.5 mCi mg^{-1}). After further incubation (37 °C, 1 h) and washing with PBS, the bottom of each well was cut out and radioactivity determined.

doses of street rabies virus, whereas mice similarly immunized with wild-type VV alone were not protected (Table 2).

To assess the authenticity of the recombinant rabies glycoprotein, reactivity with a panel of monoclonal antibodies directed against rabies glycoprotein and other viral proteins (N, NS and M) was examined. The binding activity of the recombinant glycoprotein with 44 anti-glycoprotein monoclonal antibodies was almost identical to that observed with purified ERA rabies virus, whereas only the ERA virus reacted with anti-N, -NS and -M antibodies (Fig. 3). This demonstrates that the rabies glycoprotein produced by VVTGgRAB virus-infected cells is qualitatively indistinguishable from the native glycoprotein of ERA virus.

Vaccinia virus has been used extensively as a live vaccine to control and eradicate smallpox (see ref. 13 for review); it has been developed as a cloning and expression vehicle for hepatitis B, influenza and herpes antigens and protection has been achieved by vaccination with appropriate influenza- and herpes-VV recombinants[2-4,14]. We demonstrate here that live VV expressing the rabies glycoprotein is capable of conferring protection against experimental rabies infection. Attenuated viruses such as VV are particularly appropriate vehicles for vaccine production: their preparation and administration can avoid costly procedures involving propagation of the pathogenic agent on cultured mammalian cells and subsequent toxicity testing.

We thank A. Kirn and D. Nayak for helpful discussions and P. Chambon, E. Eisenmann and P. Kourilsky for encouragement and critical reading of the manuscript, A. Balland for preparing the synthetic oligonucleotides used in this work, D. Villeval and F. Jaeger for verifying constructs by sequencing and E. Chambon and F. Daul for assistance in preparing this manuscript. This study was supported in part by NIAID grant AI-09706.

Received 27 July; accepted 27 September 1984.

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Molecular cloning and characterization of the HTLV-III virus associated with AIDS

Beatrice H. Hahn, George M. Shaw, Suresh K. Arya, Mikulas Popovic, Robert C. Gallo & Flossie Wong-Staal

Laboratory of Tumor Cell Biology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, USA

We recently reported the isolation and characterization of a novel human T-lymphotropic retrovirus, HTLV-III, in patients with acquired immune deficiency syndrome (AIDS) and in those at risk for the disease[1-4]. After extensive sero-epidemiological studies[2,3,5], together with numerous virus isolations from these patients[1,7], we concluded that HTLV-III is the causative agent of AIDS. Here we report the molecular cloning and characterization of two highly related but distinct forms of the HTLV-III genome. The viral genome is ~10 kilobases long and is detected in HTLV-III-infected cells but not in uninfected cells, including normal human tissue,

indicating that this virus is exogenous to man. We also demonstrate distant nucleic acid sequence homology between the cloned genome of HTLV-III and those of HTLV-I and HTLV-II. The availability of the cloned HTLV-III genome will now allow an unambiguous comparison of this virus with other retroviruses that also have been associated with the pathogenesis of AIDS[8-11], and moreover, will facilitate the development of diagnostic and therapeutic measures in the treatment of AIDS.

All human retroviruses that have been extensively characterized are lymphotropic, especially OKT4 lymphotropic, and induce formation of multinucleated cells on infection. These viruses also contain a relatively high-molecular weight reverse transcriptase with preference for Mg^{2+} and possess a major core protein of relative molecular mass 23,000–25,000. We named the viruses human T-cell leukaemia viruses, or HTLV, in accordance with recent convention[12,13]. The first two subgroups of HTLV (I and II) are associated with T-cell malignancies and can transform T cells *in vitro*[12]. HTLV-III has many properties in common with HTLV-I and HTLV-II but has cytopathic rather than transforming activity. The crucial step allowing us to isolate and characterize HTLV-III, and to produce sufficient purified viral reagents for serological assays, was the successful transmission of HTLV-III to an immortalized human T-cell line (HT) and to clones derived from this line which were significantly resistant to the cytopathic effects of the virus. This led to the establishment of permanently infected, high-producer cell lines for continuous production of HTLV-III[2]. One of these cell lines, H9/HTLV-III, produces large quantities of HTLV-III and serves as the principal producer cell line for immunological assays used to detect virus-specific antigens and antibodies in sera from AIDS patients. The uninfected parental cell line (HT) and its derivatives (H9 and H4) were negative by all criteria for retrovirus infection, including HTLV-I, HTLV-II and HTLV-III (M.P., in preparation). To clone the HTLV-III genome, we isolated unintegrated viral DNA after acute infection of H9 cells with concentrated HTLV-III and cloned this DNA into a λ phage library to be screened with viral cDNA.

Concentrated virus from the H9/HTLV-III cell line was used to infect fresh uninfected H9 cells at a multiplicity of 50 viral particles per cell and cultures were collected after 4, 10, 15, 24 and 48 h. Extrachromosomal DNA was extracted according to the procedure of Hirt[14] and assayed for its content of unintegrated viral DNA using HTLV-III cDNA as a probe. The synthesis of this cDNA was primed with oligo(dT) and reverse-transcribed from poly(A)-containing RNA of virions that had been banded twice on sucrose density gradients[15]. Unintegrated linear viral DNA was first detected after 10 h and was also present at the subsequent time points. Figure 1 shows a Southern blot of the 15-h sampling. A band of ~10 kilobases (kb) in the undigested DNA represents the linear form of unintegrated

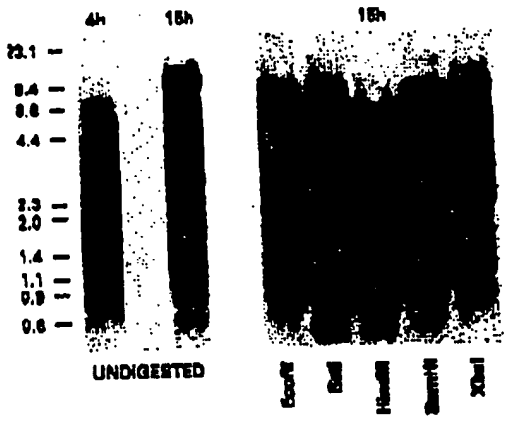

Fig. 1 Southern blot[22] analysis of unintegrated HTLV-III DNA. No viral sequences could be detected in the undigested DNA after 4 h. However, a major species of viral DNA ~10 kb long was present at 10, 15, 24 and 48 h, representing the linear unintegrated form of the virus. The figure shows a representative Southern blot of the 15-h sample digested with several restriction enzymes.
Methods: Fresh uninfected H9 cells (3×10^6) were infected with concentrated supernatant from cell line H9/HTLV-III containing 4×10^{11} particles of HTLV-III. Infected cells were divided into five roller bottles and collected after 4, 10, 15, 24 and 48 h. Low-molecular weight DNA was prepared using the Hirt fractionation procedure[14] and 30 μg of undigested and digested DNAs were separated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to an HTLV-III cDNA probe for 24 h at 37 °C in 2.4 × SSC, 40% formamide and 10% dextran sulphate. cDNA was synthesized from poly(A)-selected RNA prepared from doubly banded HTLV-III virus in the presence of oligo(dT) primers[23]. Filters were washed in 1 × SSC at 65 °C.

HTLV-III. No closed or nicked circular DNA could be detected at 10, 15 or 24 h, but both forms were evident in small amounts at 48 h (data not shown). The viral genome was not cleaved by *Xba*I, whereas *Sst*I generated three predominant bands of 9, 5.5 and 3.5 kb (Fig. 1). We interpreted these bands as representing the genomes of two forms of HTLV-III, both cut by *Sst*I in or near the long terminal repeat (LTR), and one having an additional *Sst*I site in the middle of its genome. The other enzymes generated a more complex pattern of restriction fragments requiring cloned DNA for further analysis.

Figure 2 shows the restriction map of three clones, designated λBH10, λBH9 and λBH8, which correspond in size to the three *Sst*I fragments shown in Fig. 1. Comparison of these maps suggests that λBH9 plus λBH8 constitute one HTLV-III

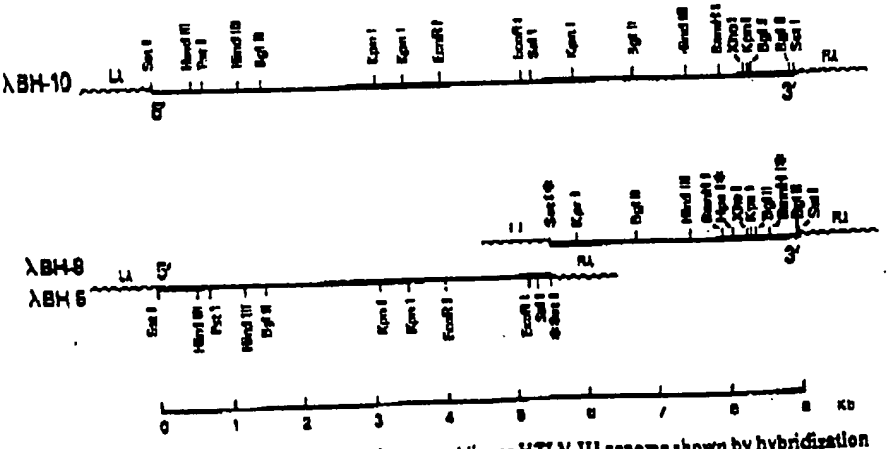

Fig. 2 Restriction endonuclease map of two closely related HTLV-III forms cloned from unintegrated viral DNA. Three recombinant clones (λBH10, λBH9 and λBH8) were analysed and their inserts (9, 5.5 and 3.5 kb, respectively) were mapped with the indicated enzymes. Together they represent two genomic equivalents of HTLV-III that are highly related but differ in three enzyme sites, indicated by bold letters and asterisks.
Methods: Low-molecular weight DNAs pooled from the 15- and 24-h samples were fractionated on a 10–40% sucrose gradient[23]. Aliquots of the fractions were electrophoresed on a 0.5% agarose gel, transferred to nitrocellulose paper and hybridized to HTLV cDNA in conditions described in Fig. 1 legend. Fractions containing the unintegrated linear HTLV-III genome shown by hybridization were pooled; the DNA was subsequently digested with *Sst*I, then ligated to phosphatase-treated *Sst*I arms of λgtWes·λB. After *in vitro* packaging, recombinant phages were screened for viral sequences with HTLV-III cDNA[17,23].

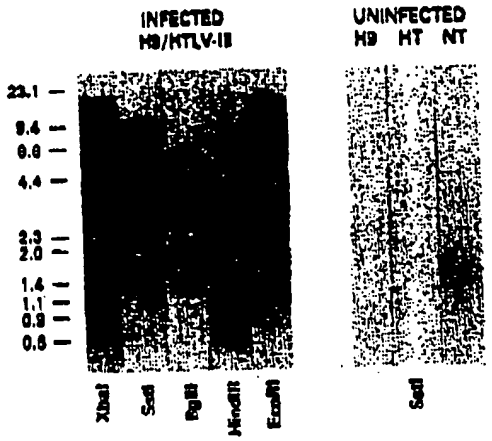

Fig. 3 Demonstration of the presence of HTLV-III viral sequences in the infected cell line, H9/HTLV-III. Both variant forms of HTLV-III defined by differences in *Sst*I sites were detected in H9/HTLV-III DNA. No HTLV-III sequences were found in uninfected H9 cells, uninfected HT cells or normal human thymus (NT). Methods: High-molecular weight DNA (10 μg) was digested with restriction enzymes as indicated and hybridized to the nick-translated phage insert from λBH10 in the conditions described in Fig. 1 legend.

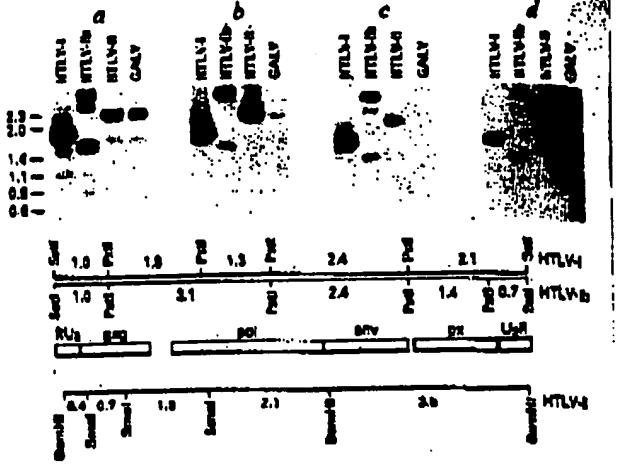

Fig. 4 Sequence homology of HTLV-III to other members of the HTLV family. Schematic restriction maps of HTLV-I, HTLV-Ib and HTLV-II are shown at the bottom, indicating the length (in kb) and location of the generated fragments with respect to the corresponding genomic regions of HTLV-I. LTR, *gag*, *pol*, *env* and pX regions are drawn to scale according to the published nucleotide sequence of HTLV-I[24]. The bands that are most highly conserved as stringency increases correspond to the *gag*/*pol* junction region of HTLV-I (1.8-kb *Pst*I fragment) and HTLV-Ib (3.1-kb *Pst*I fragment) and to the 3′ part of the *pol* region of HTLV-II (2.1-kb *Sma*I/*Bam*HI fragment) which do not overlap assuming that HTLV-II has a genomic organization similar to that of HTLV-I. Fragments corresponding to pX of HTLV-I (2.1-kb *Sst*I/*Pst* fragment) and HTLV-Ib (1.4-kb *Pst* fragment) are only faintly visible at $T_m - 28$ °C on the original autoradiogram. Digestion of GaLV generates six fragments, none of which hybridizes with HTLV-III in medium or high stringency conditions ($T_m = -42$ °C and −28 °C).

Methods: Subclones of full-length genomes of a prototype HTLV-I (unpublished), HTLV-Ib[16], HTLV-II[25] and GaLV (Seato strain)[26] were digested with the following enzymes: *Pst*I plus *Sst*I (HTLV-I and HTLV-Ib); *Bam*HI plus *Sma*I (HTLV-II); and *Hin*dIII, *Sma*I and *Xho*I (GaLV). Four replicate filters were prepared and hybridized for 36 h under low stringency (8 × SSC, 20% formamide, 10% dextran sulphate at 37 °C) to nick-translated insert of λBH10. Filters were then washed in 1 × SSC at different temperatures: *a*, 22 °C ($T_m - 70$ °C); *b*, 37 °C ($T_m - 56$ °C); *c*, 50 °C ($T_m - 42$ °C); and *d*, 65 °C ($T_m - 28$ °C), and subsequently autoradiographed for 24 h.

genome, and λBH10 another. The two viral forms differ in 3 of 21 mapped enzyme sites, including the internal *Sst*I site. As expected, the phage inserts of λBH5 and λBH8 hybridize in high-stringency conditions ($T_m - 25$ °C) to λBH10 but not to each other, as analysed by Southern blot hybridization and electron microscopic heteroduplex analysis (data not shown). To determine the orientation of the three clones, we used as a probe a cDNA clone (C15) containing U3 and R sequences (S.K.A. *et al.*, in preparation); C15 hybridized strongly to the 0.5 kb *Bgl*II fragment of λBH10 and λBH8, orienting this side 3′. Assuming that *Sst*I cuts only once in the vicinity of the HTLV-III LTR, the clones λBH10 and λBH5/λBH8 represent two complete genomic equivalents of the linear unintegrated form of HTLV-III that vary in three restriction enzyme sites. However, the viral fragments cloned into λBH5 and λBH8 may have been derived from the same or two different viruses.

The presence of two variant forms of HTLV-III in the original cell line was demonstrated by hybridizing the radiolabelled insert of λBH10 to a Southern blot of H9/HTLV-III genomic DNA digested with several restriction enzymes (Fig. 3): both forms were detected using *Sst*I, which generated the expected three bands of 9, 3.5 and 3.5 kb. *Xba*I, which does not cut the provirus, generated a high-molecular weight smear representing polyclonal integration of the provirus, plus a band of ~10 kb. This 10-kb band was also detected in undigested H9/HTLV-III DNA (not shown), indicating that it represents unintegrated viral DNA. The presence of unintegrated viral DNA also explains the 4- and 4.5-kb *Eco*RI fragments seen in both the Hirt and total cellular DNA preparations (Figs 1, 3). Both *Bgl*II and *Hin*dIII cut within the LTR and generate the expected internal bands. Several faint bands in addition to the expected internal bands generated by *Hin*dIII digestion, represent either defective proviruses or other variant forms of HTLV-III present in low copy number.

The absence of HTLV-III sequences from the DNA of the uninfected H9 cell line, the uninfected parental cell line HT and normal human thymus (Fig. 3), demonstrates clearly the exogenous nature of HTLV-III and shows that the virus does not contain human cellular sequences. The same results were obtained using inserts from λBH5 and λBH8 as probes.

The availability of the cloned HTLV-III genome also allowed us to evaluate sequence homology between HTLV-III and other members of the HTLV family including HTLV-I and HTLV-II, as well as a variant of HTLV-I (HTLV-Ib) recently isolated and molecularly cloned from a Zairian patient with adult T-cell leukaemia[16]. Replicate Southern blots of restriction enzyme-digested clones comprising the complete genomes of HTLV-I, HTLV-Ib and HTLV-II, and of gibbon ape leukaemia virus (GaLV) as a control, were hybridized with the full-length HTLV-III probe (BH10) in relaxed conditions, after which the filters were washed in conditions of low, medium and high stringency (Fig. 4). This experiment demonstrates homology between HTLV-III and HTLV-I, HTLV-Ib and HTLV-II, but not between HTLV-III and GaLV. Hybridization of HTLV-III with other members of the HTLV family could be detected in conditions ($T_m - 42$ °C and −28 °C) where no hybridization to GaLV was seen (Fig. 4*c*, *d*). Note that the restriction fragments showing greatest homology to HTLV-III correspond to the *gag*/*pol* region of HTLV-I and to an apparently non-overlapping portion of the *pol* region of HTLV-II (assuming that the genomic arrangement of HTLV-II is similar to that of HTLV-I). Hybridization to a fragment containing exclusively pX sequences in HTLV-Ib (1.4-kb *Pst*I fragment) and to the corresponding fragment in HTLV-I containing pX and LTR sequences (2.1-kb *Pst*I/*Sst*I) was detectable at $T_m - 28$ °C but was very faint. pX sequences of HTLV-II did not hybridize to the HTLV-III probe in the same stringency conditions, nor did fragments containing LTR or envelope sequences of both HTLV-I and HTLV-II.

Overall, these findings using the cloned HTLV-III probe agree with our previous observations using HTLV-III cDNA[15], which also revealed sequence homology, especially in the *gag/pol* regions of the HTLV-I, HTLV-II and HTLV-III genomes. However, we emphasize that HTLV-III is much less closely related to HTLV-I and HTLV-II at the nucleic acid level than HTLV-I and HTLV-II are to each other[17,18] and that this homology is most evident in the *gag/pol* region of these viruses under stringent hybridization.

Thus, we have molecularly cloned two closely related but distinguishable genomic equivalents of HTLV-III from the H9/HTLV-III cell line, which has been the principal source for all viral reagents used in studies of the sero-epidemiology of HTLV-III in AIDS patients[1-7]. Note that this virus from the H9/HTLV-III cell line retains its cytopathic activity against fresh normal human lymphocytes (unpublished data). Using these clones as probes, we also detected HTLV-III viral sequences in infected cell lines other than H9/HTLV-III that were established from different AIDS patients, and in fresh uncultured lymphoid tissues of AIDS patients[19]. These findings suggest that the cloned HTLV-III genomes reported here represent the probable aetiological viral agent of AIDS. The finding of two variant forms of HTLV-III in the H9/HTLV-III cell line could reflect cumulative *in vitro* mutations in a highly replicative virus. The two forms could also represent different isolates as, when first established, the H9/HTLV-III cell line was infected with pooled material from several different AIDS patients[2]. Preliminary studies of other HTLV-III isolates indeed indicate that HTLV-III, unlike HTLV-I and HTLV-II, exhibits substantial diversity in its restriction enzyme cleavage pattern when isolates from different patients are compared[19]. Further characterization and sequence analysis will help to define the natural variability of this virus, which has important implications with respect to its pathogenicity and origin, and attempts at preventive measures for AIDS. The availability of the cloned HTLV-III genome should also now allow direct comparison of this virus with a similar group of retroviruses described by other investigators[8-11] which has also been linked to the pathogenesis of AIDS and which appears to be immunologically and morphologically indistinguishable from HTLV-III (M. Samgadharan *et al.*, unpublished). Finally, the demonstration of a substantial amount of unintegrated viral DNA in the chronically infected cell line H9/HTLV-III, distinguishes HTLV-III from HTLV-I, HTLV-II and most other retroviruses. It will be important to determine whether the presence of unintegrated DNA has a role in the cytopathicity of HTLV-III, as has been proposed for certain other retroviruses[20,21].

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Metabolic oxidation phenotypes as markers for susceptibility to lung cancer

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That bronchial carcinoma is not an inevitable consequence of cigarette smoking has stimulated the search for host factors that might influence the susceptibility of the individual smoker. One plausible host factor would be a polymorphic gene controlling the metabolic oxidative activation of chemical carcinogens, giving rise to wide inter-subject variation in the generation of cancer-inducing and/or promoting species. Recently, three genetic polymorphisms of human metabolic oxidation have been demonstrated (as characterized by debrisoquine, mephenytoin and carbocysteine), with the metabolism of several substrates exhibiting the phenomenon[1–3]. Debrisoquine 4-hydroxylation segregates into two human phenotypes, each comprising characteristic metabolic capability[4–6]. We report here the frequency of debrisoquine 4-hydroxylation phenotypes in age-, sex- and smoking history-matched bronchial carcinoma and control patients. Cancer patients showed a preponderance of probable homozygous dominant extensive metabolizers (78.8%) with few recessive poor metabolizers (1.6%) compared with smoking controls (27.8% and 9.0% respectively). We conclude that the gene controlling debrisoquine 4-hydroxylation may be a host genetic determinant of susceptibility to lung cancer in smokers and that it represents a marker to assist in assessing individual risk.

The metabolism of debrisoquine was examined in 479 cigarette smokers who had or had not presented with bronchogenic carcinoma, in order to determine the frequency of extensive metabolizer (EM) and poor metabolizer (PM) phenotypes in each group. Patients were recruited from areas of London within the Islington District, Bloomsbury District and Wandsworth District Health Authorities and were admitted primarily to Chest Unit beds at Whittington Hospital. All were white Europeans with a positive history of cigarette smoking (>20 pack-yr, that is, number of packs of 20 cigarettes per day × number of years of smoking). Subjects were excluded if chemotherapy or drugs known to interfere with the phenotyping test[1] had been given, if there were signs of abnormal hepatic or renal function and if additional acute conditions such as heart failure or severe chest infection obtained. The cancer patients ($n = 245$) had a definite diagnosis of bronchogenic carcinoma proven by histology (108), cytology (85) or histology/cytology (44) from samples obtained at bronchoscopy (194), transcutaneous needle biopsy (24), mediastinoscopy (9) and pleural biopsy (6). Cell types comprised squamous cell (138), small cell (68), large cell (8) and undifferentiated (1) carcinomas, together with 30 adenocarcinoma patients. Control patients ($n = 234$) were smokers with chronic airflow limitation, without evidence of carcinoma. Each patient received no drugs after 21.30 h the day before the test, nor for 2 h after the start of the test at 07.00 h. They were each given a 10 mg debrisoquine tablet orally; all urine was collected for the subsequent 8 h and analysed for its content of debrisoquine (D) and 4-hydroxydebrisoquine (4-HD) by electron-capture gas chromatography[7]. The metabolic ratio (urinary D/4-HD) thus determined was used to assign phenotype (EM, 0.1–12.6; PM, 12.7–100)[5]. Routine clinical chemistry and haematology were performed on a blood sample from each patient within 2 days before or after the test.

Cancer and control patients were similar in age (66.5 ± 7.4 (±s.d.) and 67.2 ± 9.3 yr respectively), sex ratio (M/F) (1.82, 1.89) and smoking history (60.3 ± 24.0, 49.4 ± 21.1 pack-yr). The results showed that the patients also had similar levels of plasma Na^+ (137 ± 6, 137 ± 4 mM in cancer and control patients, respectively), HCO_3^- (27.0 ± 5.4, 26.8 ± 4.9 mM), urea (5.0 ± 1.3, 5.1 ±

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September 16, 1985

Dr. Robert Gallo
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Dear Dr. Gallo:

I have in hand your letter of July 25, 1985 addressed to Dr. Nancy Chang regarding inventorship on the Centocor HTLV-III patents. There is no question that your collaboration was essential to the overall program and, as you know, we have on every occasion, made this fact clear.

In the case of the patent covering the assay development, our lawyers advised us that, under strict inventorship interpretation, your contribution should be referenced in the patent but that you should not appear as an inventor. These rules are quite contrary to the rules for authorship on scientific papers.

We wish to have the patent legally drawn. Anything to strengthen the patent is an advantage. If the lawyers feel your name should be added, we would be not only willing but anxious to have this accomplished.

I would be happy to discuss this matter with you or your representative or arrange to have our patent attorneys visit you in Washington. If you wish to talk to our attorney, please feel free to call David Brook of Hamilton, Brook, Smith & Reynolds directly at 617-861-6240. David does our patent work and his principal client is MIT. He is most qualified in the patent area.

Your work for the government and the community is outstanding. We have attempted to support you to our utmost in the past and will use our best efforts to support you in the future.

Yours very truly,

Hubert J.P. Schoemaker, Ph.D.
President

cc: D. Brook, Esq.
N. Chang, Ph.D.
H. Wall, Chairman

Documentary Exhibit 8
CHANG ET AL.
Interference No. 103,659